Characteristics of oncolytic vesicular stomatitis virus
displaying tumor targeting ligands

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Abstract

We sought proof of principle that tumor targeting ligands could be displayed on the surface of vesicular stomatitis virus (VSV) by engineering its glycoprotein. Here we successfully rescued VSVs displaying tumor vasculature targeting ligands. Using a rational approach we investigated various feasible insertion sites on VSV G for display of tumor vasculature targeting ligands, cyclicRGD and echistatin. We found seven sites on VSV G that tolerated insertion of the nine residue length cRGD peptide, two of which could tolerate insertion of the 49aa echistatin domain. All of the ligand-displaying viruses replicated as well as the parental virus. In vitro studies demonstrated that the VSV-echistatin viruses specifically bind to targeted integrins. Since low density lipoprotein receptor (LDLR) was recently identified as a major receptor for VSV, we investigated the entry of ligand displaying viruses after masking LDLR. This experiment showed that the modified viruses can enter the cell independently of LDLR whereas entry of unmodified virus is significantly blocked by specific monoclonal antibody against LDLR. Both parental and ligand displaying viruses displayed equal oncolytic efficacy in a syngeneic mouse myeloma model. We further demonstrated that single chain antibody fragments against tumor specific antigens can be inserted at the N-terminus of the G protein and corresponding replication competent VSVs can be rescued efficiently. Overall, we have demonstrated that functional tumor targeting ligands can be displayed on replication competent VSVs without perturbing viral growth and oncolytic efficacy. This study provides a rational foundation for the future development of fully retargeted oncolytic VSVs.
Introduction:

Vesicular stomatitis virus (VSV) is an enveloped negative strand RNA virus that belongs to the *Vesiculovirus* genus of the *Rhabdoviridae* family. VSV has the ability to infect and kill cancer cells, while sparing normal cells (1, 31, 46, 48). Exploiting this oncolytic property provides a promising alternative approach for the treatment of cancer. For disseminated cancer, virotherapy should ideally be administered systemically (9, 31, 36, 43) but this route of delivery brings its own set of problems. The major concerns for VSV virotherapy are neurotoxicity, antibody neutralization and sequestration in off-target organs, especially the liver and spleen. Many attempts have been made to address these drawbacks. To reduce the neurotoxicity, the matrix protein of VSV was mutated (38, 49) and microRNA targets (25) or picornaviral internal ribosome entry sites (4) were engineered into the VSV genome. Serum neutralization has been avoided by PEGylating the virus (15, 51) or loading onto antigen-specific T cells (40) which ultimately improved virotherapy outcomes.

To circumvent all of the above hurdles in a single step, pseudotyping VSV with other viral envelope glycoprotein is a potentially feasible approach. Recent studies demonstrated that VSV neurotoxicity could be circumvented by pseudotyping with the with surface glycoproteins of lymphocytic choriomeningitis virus (29) or measles virus (10). But the reported viruses were replication-incompetent and had substantially reduced titers compared to unmodified VSVs. For oncolytic applications, an ideal VSV should have following characteristics; (i) lacks neurotoxicity (ii) evades serum neutralization (iii) extravasates efficiently into tumor tissue and (iv) targets only tumor tissue. To be an ideal oncolytic agent, VSV therefore has to be fully retargeted. As a first step to reach that goal, we attempted to display tumor targeting ligands on a replication competent VSV. Our approach was to identify novel sites in the VSV G protein to insert and display foreign peptides without compromising viral replication kinetics or oncolytic efficacy.

Several previous attempts to insert foreign peptides in to the VSV G protein were reported purely in the interest of lentivirus targeting and purification (18, 20, 35, 54). Additionally, one previous study identified a site that could tolerate insertion of a 16 residue peptide encoding an antigenic HIV epitope (44). However, there are no previous reports of ligand display on the G protein of a replication-competent VSV. In the current study we successfully rescued recombinant vesicular
stomatitis viruses displaying functional tumor targeting ligands incorporated into their engineered G proteins. We selected cyclic RGD, a nine amino-acid integrin-binding peptide, to evaluate potential insertion sites that were identified by analysis of the VSV G crystal structure. Integrins are cell surface glycoproteins which bind to extracellular matrix components, cell-surface and soluble ligands, and are involved in transmembrane cell signaling (50). Integrins also play a major role in tumor initiation, progression and metastasis (17) which makes them attractive targets for cancer therapy. RGD binds to five αV integrins (αVβ1, αVβ3, αVβ5 αVβ6, αVβ8), two β1 integrins (α5, α8) and αIIbβ3 (22). RGD in its disulphide bonded cyclic form (CDCRGDCFC) binds more strongly to integrins than in its linear form (8). RGD has been extensively studied in targeting and killing tumor cells (16). Earlier studies demonstrated that displaying RGD peptide on the surface of oncolytic parvo-, adeno-, or measles viruses can enhance their interaction with tumor vasculature and/or tumor cells (3, 26, 34). cRGD also has been used to label gold nanoparticles to target tumor cells for destruction or imaging (6, 27). Thus, this peptide was considered a good candidate to explore possible insertion sites on the VSV glycoprotein and to target VSV. We also used echistatin, a 49 amino acid snake venom disintegrin peptide that has very high affinity towards αVβ3,α5β1 and αIIbβ3 integrins (28) to explore the possibilities of rescuing replication competent VSV. Previously echistatin was displayed on oncolytic measles viruses which were subsequently shown to target tumor vasculature (21).

Based on the results of the above studies, we further examined the feasibility of displaying very large polypeptide binding domains on VSV by fusing them to the extreme N-terminus of the G protein. We successfully inserted single-chain antibody variable fragments (scFv) against tumor specific receptors at this site and rescued antibody-displaying viruses that were still capable of normal receptor binding, infection and propagation. The demonstration that it is possible to display a variety of tumor targeting ligands on the VSV G protein may have important implications for the development of a fully retargeted oncolytic VSV platform.
Materials and Methods:

Cell culture and reagents.

BHK and MPC-11 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in 5% CO2. M36 mutant cells (11) are generous gift from Dr. Felix Randow, MRC Laboratory of Molecular Biology, Cambridge, UK and were maintained in RPMI with 10% FBS and 50 microM 2-ME. K562-αvβ3 and α5β3 cells were a kind gift from Dr. S. Blystone (Upstate Medical University, Syracuse, N.Y.). K562 cells were maintained in Iscove's Modified Dulbecco's Medium (IMDM) (Life Technologies, N.Y.) supplemented with 10% fetal bovine serum, 0.5 U/L penicillin-streptomycin, and 2 mM L-Glutamine. K562-αvβ3 and K562-α5β3 cells were maintained in the same media containing 500 μg/ml of G418 (Gibco). Anti-human integrin αvβ3 (MAB1976) was purchased from Millipore, CA, USA. Monoclonal antibodies against LDLR (6E2) are kind gift from Dr. Ross Milne, Diabetes and Atherosclerosis Laboratory, University of Ottawa Heart Institute, Canada.

Construction and generation of ligand displaying recombinant VSVs.

VSV full length plasmid, pVSV-MC11 described previously (4) was modified and VSV mutant plasmid, pVSV-Δ51-MC11 (Methionine deleted at position 51 of M protein) was created as described earlier (39). For safety concern, this plasmid, pVSV-Δ51-MC11 was used as a backbone to make all the constructs mentioned in this study. Echistatin was obtained by PCR amplification of previously described measles virus plasmids (21). Echistatin and cRGD (CDRCRGDCFC) sequences were inserted into appropriate places in the full length VSV genome by overlapping PCR. Recombinant VSVs (rVSVs) were generated as previously described (4).

Briefly BHK cells were plated at a density of 1 × 10^6 cells/ well in 6 well plates. The cells were infected with vaccinia virus encoding T7 polymerase at a multiplicity of infection (MOI) of 10. After an hour vaccinia virus was removed, and the cells were transfected with 1 μg pVSV, 0.5 μg pN, 0.4 μg pP, and 0.2 μg pL using 6μl of Lipofectamine LTX transfection reagent (Life Technologies, NY, USA) according to the manufacturer's instructions. The cells were incubated for 6 h at 37°C, and then the medium was replaced with DMEM with 5% FBS. After 48 h, culture medium was harvested, filtered twice through a 0.2-μm filter, and overlaid onto new BHK cells in a 6 well plate. Forty-eight hours later, the culture medium was harvested, subjected to low-speed centrifugation and titrated on fresh BHK cells. When necessary the recombinant
viruses were further passaged to amplify the viral titer. Viral titers were determined by plaque assay as described previously (41). All the recombinant viruses were verified by sequence analysis.

**Growth curves analysis.**
Growth curve analysis has been carried out as described earlier (4). For multi-step growth curves BHK cells were incubated with rVSV at an MOI of 0.01 for 1 h at 37°C. Following this incubation, supernatant was removed, the monolayer was washed and fresh growth medium was added. Supernatant was collected at predetermined time points (6, 12, 18, 24, 30 and 36h), and virus titer was determined by standard plaque assay.

**Western blot, immunofluorescence and FACS analysis.**
Western blot was done as described earlier (4). To detect viral protein expression levels, VSV-infected cells were harvested at the indicated time points, and incubated with 100 μl of RIPA buffer (25 mM Tris-HCl, [pH 7.6], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) at 4°C for 10 min. Cell lysate was centrifuged, and the supernatant was collected and stored at -20°C until used. For immunoblotting, the proteins were electrophoretically separated in a 12% sodium dodecyl sulfate-polyacrylamide gel. VSV proteins were detected by western blotting using polyclonal antibody against wild-type VSV and echistatin was detected using polyclonal serum against echistatin peptide (Millipore, MA, USA). Immunofluorescence microscope was used to analyze and image GFP expressing cells. We used ImageJ software (http://rsbweb.nih.gov/ij/) to quantify GFP positive cells. The surface expression level of integrin was detected by FACS analysis. M36 cells were washed twice with PBS and fixed with 4% paraformaldehyde. Cells were blocked with 2% Horse serum in 20 min at room temperature. Then incubated with anti-α5β1 antibody (Millipore, MA, USA) followed by secondary antibody 1 hr each at room temperature. After final washing with PBS, the cells were analyzed by flow cytometry using a FACScan system with CELLQuest software (Becton Dickinson).

**Virus binding and quantitative RT-PCR.**
Binding assays, using wild type and echistatin displaying viruses, were carried out in suspension as described earlier (5, 23). Briefly K562 cells were pelleted and washed with PBS three times. Final cell pellet was dissolved in PBS, counted and aliquoted into different tubes. Cells and
viruses (MOI=0.1) were incubated for 2 h at 4°C or 30 min at 37°C. Cells were washed three times in cold PBS to remove unbound viruses. Then cell pellets were stored at -80°C for further processing. The cells were lysed in 400 μl RLT buffer (Qiagen, CA, USA) and total RNA was then extracted using the Qiagen RNase kit® following the manufacturer’s instructions. Quantitative two-step RT-PCR was performed according to manufacturer’s instructions (LightCycler® 480 Real-Time PCR System, Roche Applied Science, IN, USA). For reverse transcription random hexamer was used and followed by quantitative real time PCR was carried out using primers directed against N gene. Standard curve was made using serial dilutions of known virus genome. The copy numbers for the samples were calculated by Light Cycler 480 software.

In vivo experiments.
All animal protocols were reviewed and approved by the Mayo Clinic Institutional Care and Use Committee. BALB/c mice, female, 4-6 weeks old, were purchased from Jackson Laboratories. Mice were implanted with 5 × 10⁶ mouse plasmacytoma (MPC-11) cells in the right flank. When tumors reached an average size of 0.2 - 0.5 cm³, mice were treated by single intravenous injection of recombinant VSV. Tumor volume was measured using a hand-held caliper. The mice were monitored daily until the end of the study or when they reach euthanasia criteria. Euthanasia criteria: Clinical signs of neurotoxicity, tumor ulceration, tumor volume more than 10% of body weight, greater than 10% weight loss, or if mice are unable to gain access to food or water.

Immunohistochemistry.
Tumors harvested were frozen in optimal cutting medium (OCT) for sectioning. Tumor sections were analyzed by immunofluorescence for VSV antigens using polyclonal rabbit anti-VSV, and for endothelial cells using anti-CD31 (Abd serotec, NC, USA) followed by Alexa-labeled anti-rabbit IgG secondary antibody (Life Technologies, NY, USA) and cellular nuclei using Hoescht 33342 (Life Technologies).

Statistical analyses.
An unpaired two-tailed Student t test was carried out to compare the values. Survival curve analysis has been done using GraphPad Prism 4.0 program (GraphPad Software, San Diego,
Survival curves were plotted according to the Kaplan-Meier method, and survival function across treatment groups was compared using log-rank test analyses.

**Results:**

**Construction and rescue of VSVs displaying tumor targeting peptides.**

To identify possible sites within the VSV G ectodomain that might tolerate insertion of a foreign peptide while allowing rescue of viable virus, we analyzed and compared amino acid sequences of various vesiculovirus G proteins. The crystal structure of the pre-fusion form of VSV G was also used to predict feasible insertion sites. Three types of site were identified as being more likely to tolerate insertion; highly variable regions, regions rich in hydrophilic residues, and loop regions. Regions showing high variability between homologous rhabdovirus G proteins were predicted to be more likely dispensable for protein stability and function. Foreign peptides inserted between hydrophilic residues were expected to be better exposed on the protein surface. And loop regions are usually flexible, hence more likely to allow insertion of epitopes without disturbing protein folding and structure. To display the cyclic RGD peptide on VSV G, we fused the CDCRGDCFC sequence in-frame into the G protein in a full-length viral genome carrying enhanced green fluorescent protein (EGFP) as a marker gene (Fig. 1A). The amino acid numbering used in this study is taken from the VSV G crystal structure (42). Figure 1B-1D shows the tested RGD insertion sites on the VSV G protein. Seven of the thirteen sites allowed recovery of viable viruses (Fig. 2A) which could be efficiently amplified in BHK cells. Since our goal was to insert larger peptides into those sites, we focused on the four RGD insertion sites that gave rise to viruses that replicated with wild-type virus kinetics. We then replaced cRGD with the 49 amino acid long echistatin peptide in those four constructs. Among four constructs, two of them yielded viable viruses; one with echistatin inserted at the N-terminus of G next to the signal peptide and another one with an insertion between amino acids 351T and 352T (Fig. 2A).

Wild-type and ligand displaying viruses were analyzed by immunoblotting for viral structural proteins (Fig. 2B). Very slight variations were noticed in the mobility of the G protein among the RGD displaying viruses. In the case of echistatin display, the G protein of the VSV-Echi1 virus had significantly faster mobility than that of VSV-Echi9. Sequence analysis of the G genes of all...
the rescued viruses showed intact ligand sequences. We remain uncertain as to what causes this mobility difference between the G proteins of the two echistatin displaying viruses. Since echistatin in the VSV-Echi1 virus is immediately adjacent to the signal peptide, there may be a cryptic signal peptidase cleavage signal in the echistatin peptide. Alternatively, there may be a difference in the glycosylation status of the two chimeric G proteins. When the membrane was probed with anti-echistatin polyclonal antibody, both Echi1 and Echi9 virus glycoproteins were efficiently recognized (Fig. 2B). Multi-step growth curve analysis showed that all of the recombinant viruses had replication kinetics similar to the parental virus (Fig. 2C) although some of them were slightly inferior. Since VSV-Echi9 displays an intact echistatin peptide and grows comparatively better than VSV-Echi1 virus, we used VSV-Echi9 virus for most of our subsequent studies.

**Virus neutralization assays.**

A virus neutralization assay was performed to determine whether antibodies against echistatin are able to neutralize the infectivity of echistatin-displaying VSV. Recombinant VSVs (10^3 PFU) were incubated with serial dilutions of either anti-echistatin or anti-VSV serum before plating onto BHK cells. VSV displaying echistatin was effectively neutralized by anti-echistatin serum which had no effect on wild-type virus infectivity (Fig. 3A). This suggests that the echistatin peptide is exposed on the surface of virion. On the other hand anti-VSV serum neutralized both the viruses (Fig. 3B).

**Solid-phase receptor-binding assay.**

To determine whether echistatin displaying VSV can bind to its specific receptor, we coated microtiter wells with purified integrins. The viruses were allowed to bind to the immobilized integrins and after 1hr of incubation unbound viruses were washed away with PBS. The bound viruses were rescued by addition of MPC-11 cells to the microtiter wells. The VSV-Echi9 virus bound significantly better to integrin, αvβ3 than to the control integrin, α1β1 (Fig. 4). This study shows that a ligand-displaying VSV has the ability to bind to its targeted receptor thorough the displayed ligand and the binding is highly specific.
Infection of GP96 mutant (VSV receptor negative) cells by ligand displaying viruses.

In a recent study Bloor and co-workers (12) demonstrated that cells without the endoplasmic reticulum chaperone GP96 or with a catalytically inactive gp96 do not bind VSV-G. When the GP96 mutant M36 cells were infected with unmodified and recombinant VSVs, the ligand displaying viruses (especially VSV-Ech9 and VSV-RGD9) showed significantly higher infection compared to unmodified virus (Fig. 5). Since the M36 cells do not express αvβ3 and αIIβ3 integrins (47, 52), we postulated that the ligand-displaying viruses might be entering through α5β1. We therefore analyzed M36 cells by flow cytometry, and confirmed a low level expression of α5β1 (Fig. 5C), consistent with the hypothesis that VSV-Ech9 and VSV-RGD9 viruses may bind the cells through this receptor. However infectivity of the ligand displaying viruses with different insertion sites was not significantly higher compared to the unmodified VSV. This observation warrants further detailed study to determine whether the precise positioning of the displayed ligand plays a role in receptor affinity.

Specific binding of echistatin displaying VSV to cells over-expressing targeted integrins.

We next investigated whether the virally displayed echistatin domain could mediate specific viral binding to cells over expressing the targeted integrin, αvβ3. For this we incubated K562 (human erythroleukemia) cells with VSV-WT and VSV-Ech9 for 30min at 37°C and also at 4°C for 2h. After washing, the bound virus particles were subjected to RNA extraction and quantitative RT-PCR. Analysis showed that affinity of unmodified and echistatin virus towards K562 wild-type cells were almost the same (Fig. 6A). On K562-αvβ5 cells, the affinity of VSV-Ech9 was slightly higher than unmodified VSV (Fig. 6B). At the same time affinity of VSV-Ech9 to K562-αvβ3 cell surface increased significantly compared to unmodified virus (Fig. 6C). It shows that VSV displaying echistatin has the ability to bind to is specific receptor in this case αvβ3, with high affinity.

Infection via the targeted receptors in vitro.

We next investigated the entry of ligand displaying VSVs in human cell lines expressing integrins after masking one of the major VSV receptors (19). To this end, we blocked low density lipoprotein receptor (LDLR) with a specific monoclonal antibody against human LDLR (19, 33). K562-αvβ3 cells were incubated with equal amounts of anti-LDLR monoclonal
antibody for an hour at 37°C. Then VSV (10^3 PFU) was added and incubated for 30 min at 37°C, cells were washed twice with PBS, growth medium was added and 16 h later infected cells were analyzed for GFP expression to calculate virus titer (Fig. 7A-C). Since wild-type has a minor growth advantage over the ligand displaying viruses, it gives a slightly higher titer in antibody untreated cells compared to other viruses (Fig. 7B). Conversely, in anti-LDLR antibody treated cells, the infectivity of wild-type VSV was significantly blocked whereas the ligand displaying viruses (especially VSV-Echi9 and VSV-RGD3) retained their infectivity.

To further confirm that the ligand displaying VSVs could infect via an integrin receptor, we treated the K562-avß3 cells with a functional blocking anti-avß3 monoclonal antibody in addition to the anti-LDLR antibody. Individually, neither antibody could significantly inhibit the infectivity of the VSV-Echi9 virus, but in combination they could. Conversely, the infectivity of the wild-type virus was efficiently blocked by the LDLR antibody but not by the anti-avß3 antibody (Fig. 7D-E). This experiment strongly suggests that the echistatin displaying VSV can enter cells via the targeted integrin receptor even when the LDL receptor is effectively masked.

**Oncolytic activity of targeted viruses in vivo after intravenous administration.**

We next evaluated the oncolytic activity of the recombinant VSVs in mice with established syngeneic plasmacytomas (MPC-11). Compared to unmodified VSV, the RGD and echistatin displaying viruses showed similar therapeutic potency. No significant difference in oncolytic efficacy was observed (Fig. 8). Interestingly the Echi-1, RGD-3 and RGD-7 viruses cured all treated mice, whereas a single relapse was seen in groups of five mice treated with WT, Echi-9 and RGD-9 viruses, and two relapses in the group treated with RGD-1 virus. One tumor spontaneously subsided in the untreated control group. In a separate study tumors were harvested 24 h post intravenous administration of viruses for immunohistochemical analysis (Fig. 9A). Since tumor blood vessels express high levels of integrins, the RGD and echistatin viruses were expected to infect the blood vessels. Dual staining using anti-mouse CD31 and anti-VSV polyclonal antibody was therefore performed to identify endothelial cells of tumor blood vessels and VSV infection respectively. The staining showed no difference between unmodified and ligand displaying VSVs. Figure 9B shows uninfected tumor blood vessels surrounded by VSV infected cells.
Rescue of recombinant VSVs displaying single chain antibodies.

To determine whether larger polypeptide ligands could be displayed on VSV as G protein fusions, we fused single chain fragment variables (scFv) against epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2), at the N-terminus of the glycoprotein (Fig. 10A). Even though initial virus recovery was slow, multiple passages (~5) yielded high titer viruses. Sequencing results showed that the nucleotide sequence of the displayed scFv domains was fully intact in the passaged viruses, with a few nucleotide changes in the underlying G protein which may have been selected for better accommodation of the larger peptides (data not shown). VSVs displaying scFvs achieved lower maximum titers (by one to two orders of magnitude) than unmodified VSV on BHK cells (Fig. 10B). Western blotting showed that the EGFR-scFv was partially cleaved from the G protein whereas the Her2-scFv-G protein fusion remained fully intact with no cleavage (Fig. 10C). Since VSV can adapt during replication in cell culture, additional passages may further increase its replication potential and also codon optimization of insert may be necessary to obtain high replicating viruses.

Discussion

Vesicular stomatitis virus is a rhabdovirus with inherent oncolytic properties which effectively kills cancer cells while sparing normal cells (14). A phase I clinical trial was recently initiated at Mayo Clinic in which VSV is being given as experimental intratumoral therapy to patients with hepatocellular carcinoma. But for future trials of intravenous VSV therapy it will be important to engineer the virus for greater safety and efficacy. To achieve this goal it will be important to develop oncolytic VSVs that are highly tumor-specific, free of neurotoxicity and at that can clear all the hurdles (neutralization, sequestration and lack of extravasation) of systemic administration (43). Towards this goal, and with a view to retargeting the virus, we sought to display tumor targeting ligands on the surface of virion. Our data demonstrate that it is feasible to insert peptides as large as single chain antibody fragments into the G protein and rescue replication competent VSVs which display the inserted domains on their surface.
Previously it has been shown that peptides up to 16 amino acids in length could be inserted into a permissive site on the VSV G protein of a replication competent VSV (44). To our knowledge, no other successful attempt to display a ligand on a replication competent VSV has been reported. Since our laboratory has had some success targeting other virus types for the purpose of oncolytic virotherapy (21, 24, 32), we decided to explore the feasibility of targeting VSV by displaying tumor targeting ligands on its G protein.

We initially selected the integrin-binding cyclic RGD (cRGD) peptide to explore feasible insertion sites in the VSV G protein because of its smaller size and ability to target tumor blood vessels. The RGD motif has been displayed previously on the surfaces of many viruses [measles virus (34), parvovirus (2), adenovirus (30, 37, 53), adeno-associated virus (45)] with the goal of enhancing their oncolytic specificity and/or transduction efficiency. In selecting potential insertion sites for the VSV G protein, we relied heavily on the crystal structure of the protein and sequence comparisons with the G proteins of various related vesiculoviruses. Using that information we identified 13 potential sites in VSV G, seven of which could tolerate the nine amino acid long cRGD insertion which can be displayed on the virion surface. Among these, two of the sites yielded viable viruses when sequence encoding the 49 aa long echistatin polypeptide domain was inserted (Fig. 2A). The recovery and efficient replication of the echi-9 virus was really very interesting. The echi-9 site between amino acids 351 and 352 is located in the middle of lateral domain of the VSV G protein. The fact that G could tolerate insertion of a functional domain within this domain which is not only a structural but also a functional unit was quite surprising. VSV G is a multi-domain protein comprising at least four major domains. Most multi-domain proteins are formed by a linear sequence of independently folding domains which are secured by end-to-end linkages (7). VSV G protein is an exception to this pattern exhibiting discontinuity in its domain arrangement which allows insertion of a fully functional domain within a domain, without affecting its protein folding and structure. This study suggests that one can insert any reasonable size of functional domain into this site in the VSV G protein, and probably into a corresponding site in any rhabdovirus G protein, as long as the insert does not interfere with G protein folding and maturation.
Display of the echistatin peptide on the virion surface was confirmed by neutralization assay (Fig.3). At the same time specific binding of the echistatin displaying virus to αvβ3 integrin in a solid-phase capture assay demonstrated that VSV can bind to a targeted receptor through a polypeptide ligand displayed on its surface (Fig.4). When the recombinant viruses were incubated with wild-type and integrin overexpressing K562 cells, VSV-Echi-9 bound with considerably higher affinity to K562- αvβ3 cells compared to wild-type virus (Fig.6). These data further confirmed the higher affinity of echistatin displaying VSV to its targeted receptor.

A recent publication proposed and demonstrated that low-density lipoprotein receptor (LDLR) and its family members serve as natural receptors for vesicular stomatitis virus (19). Therefore, to determine whether VSV could enter cells via a targeted receptor recognized by a displayed ligand we used an anti-LDLR monoclonal antibody to mask the LDLR on K562 cells that overexpress αvβ3 integrin (K562-αvβ3) (33). Figure 7 shows that wild-type virus infection was efficiently blocked by the LDLR monoclonal antibody, whereas the ligand displaying viruses, especially VSV-Echi9, showed significantly higher infection. Surprisingly VSV-RGD3 also showed significant infection. This study suggested that VSV can enter cells through its displayed ligand. This conclusion was further confirmed by demonstrating that infection by the echistatin displaying VSV was significantly blocked when the cells were treated both with monoclonal against LDLR and αvβ3 integrin.

The oncolytic potency of the ligand displaying viruses was tested in a mouse myeloma model. Immunocompetent Balb/C mice bearing syngeneic subcutaneous mouse plasmacytomas were treated with single intravenous dose of VSV. There was no significant difference in tumor response or survival between wild-type and recombinant viruses (Fig. 8). Since cRGD and echistatin are theoretically capable of targeting tumor vasculature, we were curious to see whether tumor blood vessels were infected with VSV. However, dual staining (αCD31 and αVSV) of tumor sections did not yield any conclusive results (Fig. 9B). Recently it was shown in a different tumor model that VSV can infect tumor vasculature directly (13), but it is not evident in our model. We did observe VSV infection immediately adjacent to the intact tumor blood vessels but not in the lumen as was observed in a previous study from our laboratory using different syngeneic myeloma model (31). Whether the displayed RGD or echistatin peptides can target VSV attachment to endothelial cells is not clear. One possible scenario is that the
displayed peptides may mediate initial binding of VSV to tumor endothelial cells which express high levels of αvβ3 integrin and thereby facilitate extravasation of the virus into the tumor parenchyma without directly infecting the vascular endothelial cells. The 100% cure of MPC11 tumors by some but not all of the targeted viruses, (Ech1-1, RGD-3 and RGD-7) may give some support to this idea but more detailed studies will be needed to confirm the impression.

We further tested the ability of the VSV G protein to tolerate large polypeptide insertions by fusing single-chain antibody variable fragments (scFv) to its N-terminus and rescuing replication competent VSV. Previous studies from our laboratory demonstrated that measles viruses displaying single chain antibodies against various cancer antigens were efficiently targeted to tumors expressing the respective cognate receptors (32). We therefore fused single chain antibodies against epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2) to the N-terminus of the G protein (Fig. 10) and successfully rescued viruses encoding the chimeric proteins. Western blot analysis shows that there is partial cleavage of the EGFR scFv from the chimeric G protein (Fig. 10) which may be due to improper folding chimera which is not apparent in the case of the HER-2 antibody displaying virus. While scFv displaying VSVs grow to a maximum titer that is one or two logs lower than the unmodified virus, insertion of a large domain at this site is very appealing. Further optimization of the system may well yield viruses with high replication potential. However, rescue of replication competent VSVs displaying intact scFvs marks a necessary milestone in vesiculovirus targeting.

Vesicular stomatitis virus has recently made the transition into the clinic from the laboratory. Although safety is prime importance for the clinical therapy, efficacy is also a key factor for successful curative therapy and should not be compromised. In the MPC11 myeloma model VSV has the ability to infect and kill most of the cancer cells before the virus gets eliminated by the immune system. Many replication competent viruses have been attenuated to the point of compromising their ability to fight innate immunity. Since even attenuated VSVs retain some neurotoxic potential when administered as systemic therapy, there is a strong rationale to modify their tropism to allow effective binding and entry via tumor-specific receptors without compromising oncolytic potency. To this end we have successfully displayed tumor targeting ligands on the surface of the G protein and have demonstrated that the modified viruses have equivalent oncolytic potency when compared to the unmodified virus. This is a proof-of-concept
study which demonstrates the feasibility of displaying tumor targeting ligands on replication
competent VSVs and suggests that full retargeting will indeed be possible if the natural receptor
tropism can now be ablated. Ligand displaying VSV G proteins might also be of interest to
retarget lentiviral vectors.

A major receptor for VSV (LDLR family) was proposed recently but there are many questions
yet to be answered. How many receptors really exist for VSV? Does VSV also use carbohydrates
or lipids for attachment? How does VSV interact with its individual receptors when more than
one is available? What is the role of receptor density and of steric hindrance for access to
competing receptors? It is also unclear whether VSV uses the same receptor(s) on all cell types
or uses a different receptor(s) on each cell line. Is there any interspecies variation in the nature or
binding affinity of the receptor? Does the absence of one receptor alter the binding affinity of
VSV towards another receptor? There are still a great many unanswered questions about VSV
receptor and this is an important area of study. We anticipate that ligand display on the VSV G
protein may open the door to find answers to some of those questions. In addition, besides
contributing to the understanding of VSV entry, this study may lead to the development of fully
targeted oncolytic vesicular stomatitis virus in the near future.

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Heart Institute, Canada, for providing anti LDLR monoclonal.
References:


**Figure Legends:**

**Fig 1. Ligand insertion sites on VSV G.** (A) Linear map of VSV genome with GFP. (B) Amino acid sequence of VSV G (crystal structure) showing cRGD insertion sites (C) Insertion site of ligands (Bold letters) at the N-terminus of G protein. Only the signal peptide (italics) and a few residues of G are shown (underlined). *An additional lysine residue was included to maintain the proper G sequence. (D) Crystal structure of VSV G protein showing cyclic RGD insertion sites. Pymol program was used to edit G crystal structure (PDB ID: 2J6J).

**Fig 2. Virus recovery and analysis of growth properties.** (A) Table shows various VSV constructs with ligand insertion sites and virus recovery (B) Western immunoblot shows G and N/P proteins of purified recombinant VSVs which were probed with anti-VSV polyclonal (upper panel) and anti-echistatin polyclonal (lower panel) (C) Multi-step growth curve analysis of indicated viruses (MOI: 0.01).

**Fig 3. Neutralization assay.** 1000 PFU of VSV-WT or VSV-Echi9 were incubated with two-fold serial dilutions of antibody against echistatin peptide (A) or wild-type VSV (B) for 1 h at 37°C before addition to BHK cells. After 30 minutes, monolayer was washed twice with PBS and was overlaid with methyl cellulose. Plaques were counted 48hrs post infection. Results are expressed as percentage of the plaques obtained with no antibody.

**Fig 4. Solid-Phase receptor binding assay.** (A) Unmodified (VSV-WT) or echistatin displaying VSV (VSV-Echi9) binding to (A) PBS (B) α1β1 and (C) αvβ3 integrin. PBS, α1β1 and αvβ3 were coated on microtiter wells (0.2 μg/ml) and incubated with either VSV-WT or VSV-echi9 (10^3 PFU). After 1hr incubation, wells were washed three times with PBS and MPC-11 cells were added to rescue bound viruses. GFP positive cells were counted using ImageJ program. Results are expressed as mean ± standard error of triplicate wells (D). Significantly higher binding was noted in αvβ3 coated wells with VSV-Echi9 (*, P = 0.0001) compared to VSV-WT.

**Fig 5. Infectivity of ligand displaying VSVs in GP96 mutant cells.** The experiment was done in triplicate with M36 cells at an MOI of 1 and 10 and analyzed after 16h post infection (A) GFP expression of infected cells at an MOI of 10.0 (B) GFP positive cells were counted using ImageJ program. (C) Flow cytometry analysis of M36 cells for α5β1expression. Significantly higher infection shown by VSV-Echi9 and VSV-RGD9 (*, P < 0.01) compared to VSV-WT.
Fig 6. Affinity of echistatin displaying VSV towards integrin over expressing cell line. Wild-type (A) or αvβ3 (B) or αvβ5 (C) over expressing K562 cells were incubated with VSV-WT and VSV-Echi9 viruses at an MOI of 0.1. After 2hr (4°C) or 30 min (37°C) of incubation, cells were washed twice with PBS and total RNA was extracted as described in materials and methods. Quantitative analysis of N gene copies was carried out using qRT-PCR. Error bars represent the standard error of the mean. Where error bars are not visible, the standard error was negligible. Significantly higher affinity shown by VSV-Echi9 (*, P < 0.01) compared to VSV-WT.

Fig 7. Receptor specificity of targeted viruses. K562-αvβ3 cells were treated with anti-LDLR and or anti-αvβ3 monoclonal for 1hr before addition of VSVs. After an hour cells were washed and incubated for 16h before measuring virus titers and imaging. (A) Infectivity in untreated (left) and in α-LDLR treated K562-αvβ3cells (right). Titers of viruses from untreated (B) and α-LDLR treated (C) K562-αvβ3cells. Significantly higher infection shown by VSV-Echi9 and VSV- RGD3 (*, P < 0.01 compared to VSV-WT) (D) Infectivity of VSV-wt and VSV-Echi9 viruses in K562-αvβ3cells treated with α-LDLR and anti-αvβ3 antibodies. (E) Titers of viruses from panel D (*, P = 0.0105 compared to titer of VSV-Echi9 from K562- αvβ3 cells treated only with α-LDLR).

Fig 8. Oncolytic efficacy of ligand displaying viruses. Mice (BALB/c, 4-weeks old, n=5) bearing subcutaneous MPC-11 tumors were treated with a single intravenous dose (5x10^6 PFU) of (A) Saline, WT and RGD and Echistatin displaying viruses. Tumor size was measured by serial caliper measurements (B) Kaplan-Meier survival curves for the mice in panel A. No significant difference was noted between VSV-WT and other ligand displaying viruses.

Fig 9. Analysis of virus spread in myeloma tumor in vivo. MPC-11 tumor bearing mice were injected with a single i.v (5x10^6 PFU) dose of indicated viruses. Tumors were harvested and sectioned at 24h post treatment and analyzed for VSV antigen (red) and cell nuclei (Hoescht/blue). Images are at 4x magnification. (B) Tumor sections were stained for VSV antigen (Green) and endothelial cell marker CD31 (Red). Tumor blood vessels seem uninfected.

Fig 10. Rescue of single chain antibody fragment variable (scFv) displaying VSVs. (A) Schematic representation of VSV genomic construct with N-terminal scFv (B) Multi-step growth
curve of scFv displaying VSVs (C) Western blot analysis of purified VSV-scFv proteins probed with anti-VSV polyclonal antibody.
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**Figure B**

- α-VSV
  - WT
  - Ech1
  - Ech9
  - RGD-1
  - RGD-2
  - RGD-3
  - RGD-7
  - RGD-9

- α-Echistatin
  - N/P
  - G

**Figure C**

- Virus titer (log10)
- Hours post infection
- Graph showing virus titer over time for different constructs and mutants.